ASSOCIATION OF RIBOSOMAL SUBUNITS: MECHANISM OF THE REACTION INDUCED BY ASSOCIATION FACTOR

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1. Introduction

The association of ribosomal subparticles into monomers is one of the general properties of bacterial ribosomes that has been more extensively investigated in the last few years.

In 1959, Tissières et al. [1] found that subunits form 30 S–50 S couples when the Mg^{2+} concentration is increased up to 10 mM or higher. These results have been confirmed by other investigators and many studies on the equilibrium between the monomer and its subunits have now been reported [2–4]. This equilibrium can also be shifted towards association by addition of polyamines [2, 5–7], some non-ionic agents such as ethanol and dioxane [8], or aminoglycoside antibiotics, such as neomycin [7, 9–11].

On the other hand ribosomal subparticles tend to associate at relatively low Mg^{2+} concentrations when they are attached to aminoacyl- or peptidyl-tRNA [12, 13].

Recently we have described a new factor which produces the *in vitro* association of washed ribosomal subunits (free of aminoacyl- and peptidyl-tRNA) at 3–5 mM Mg^{2+} [14]. This association factor (AF) has been obtained by extracting ribosomes of *Bacillus stearothermophilus* with a strong salt solution. AF will not only associate the ribosomal subunits obtained from thermophilic bacteria but also those of *Escherichia coli.*

The splitting of 70 S monomers caused by dissociation factor (DF) can be reversed by the addition of AF [14]. It has been shown that following dissociation of the 70 S ribosomes the DF remains bound to 30 S particles [9, 15, 16]. If DF has to be displaced by AF before reassociation of the subunits can occur, it is possible that the AF target is also the smaller subparticle.

In this paper we describe the interaction between AF and the ribosomal subunits, the steps in the association process and a fractionation of AF into two components.

2. Material and methods

2.1. *Culture conditions and preparation of ribosomes and AF*

E. coli D_{10} was grown at 37° in a medium M_9 [17] with the addition of Casamino acids (2 g/l). *B. stearothermophilus* 1503-4R was grown at 65° in a Bacto-Pantone yeast hydrolysate medium [18]. Both bacteria were harvested and broken as previously reported [19].

E. coli ribosomes were obtained from exponentially growing cells harvested after slow cooling, and were mostly in the form of 70 S particles [20].

The suspended ribosomes were washed and dissociated by two repeated treatments of 1 hr in a solution containing 10 mM Tris-HCl (pH 7.8) – 7.5 mM Mg acetate–1 M KCl–4 mM 2-mercaptoethanol, followed by centrifugation at 165,000 *g* for 5 hr. The final pellet was resuspended in a small volume of buffer A (10 mM Tris-HCl, pH 7.8, 5 mM Mg acetate, 50 mM KCl and 2 mM 2-mercaptoethanol) and the washed

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subunits were isolated as already described [14]. When necessary subparticles were concentrated by centrifugation at 165,000 *g* for 10 hr and resuspension in a convenient volume of buffer A.

Association factor was obtained from ribosomes of *B. stearothermophilus* harvested at the stationary phase of growth as previously reported [14]. These preparations were heated for 10 min at 80° to destroy any residual dissociating activity.

The standard assay conditions and the method used to calculate percentage of association have been detailed previously [14].

2.2. *Binding of AF to 30 S or 50 S subunits*

The reaction mixture (1 ml) contained 10 mM Tris-HCl (pH 7.8), 5 mM Mg acetate, 50 mM KCl, 2.5 mM dithiothreitol, 5–6 A_{260} units of ribosomal subparticles (30 S or 50 S) and dialyzed association factor (0.4–2 mg of protein). After incubation for 5 min at the indicated temperatures, the tubes were chilled to 0° and each sample was submitted to filtration through a Bio-Gel A-0.5 m column (1.0 × 21.0 cm) equilibrated with buffer A. The elution was accomplished with the same buffer solution; fractions containing ribosomes were detected by the absorbance at 260 nm, pooled and stored in the refrigerator. They were subsequently used in the partial reaction of association.

2.3. *Gel filtration of AF*

A preparation of AF (0.4 ml containing about 1 mg of protein) was applied to a Sephadex G-25 column (0.4 × 30 cm) previously equilibrated with buffer A. The column was washed and eluted with the same buffer and fractions corresponding to molecules either completely excluded (AF_I) or included (AF_{II}) were pooled separately and stored at −20°. (The Sephadex column was calibrated with blue Dextran and NaCl as markers for totally excluded and included substances, respectively).

3. Results and discussion

The association of ribosomal subparticles induced *in vitro* by AF could proceed either as a single step or through a complex process involving several steps. To determine whether AF binds to one of the subunits before the association, the 30 S and 50 S particles were separately incubated with AF and submitted to filtration through Bio-Gel A-0.5 m in order to eliminate the excess of free AF. The excluded fractions, containing any of the ribosomal subunits with or without AF attached to them (but no free AF) were

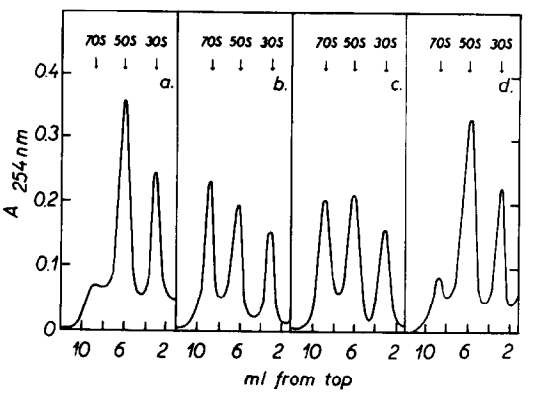

Fig. 1. Ribosomal binding of AF. Sedimentation profiles of samples obtained after association of subunits with or without previous binding of AF; (a) and (b) contained 30 S and 50 S particles incubated for 5 min at 45° in the absence and in the presence of AF (40 μg of protein), respectively. (c) 30 S subunits were preincubated at 55° with AF, and after filtration through Bio-Gel A-0.5 m, the excluded fractions were incubated for 5 min at 45° in the presence of 50 S particles; (d) as in (c), but performing the binding step with 50 S subunits, and the association after the addition of small subparticles. All other conditions were detailed in Methods.

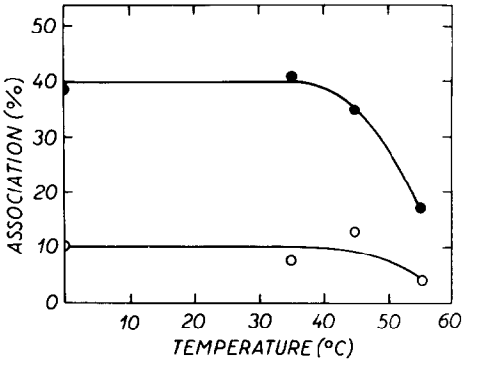

Fig. 2. Partial reaction of association at different temperatures. The binding step was performed as in fig. 1 with 30 S (●—●—●) or 50 S (○—○—○) particles and followed by association at the indicated temperatures.

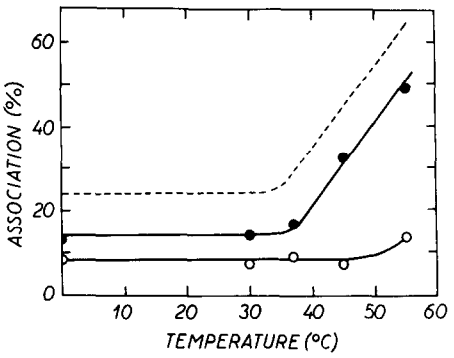

Fig. 3. Partial reaction of binding at different temperatures. Binding step carried out with 30 S subunits in the presence (●—●—●) or absence (○—○—○) of AF at the indicated temperatures was followed by association at 0°. All other conditions as in Methods. For comparison the temperature curve of the whole association process (- - - -), taken from a previous work [14], is included.

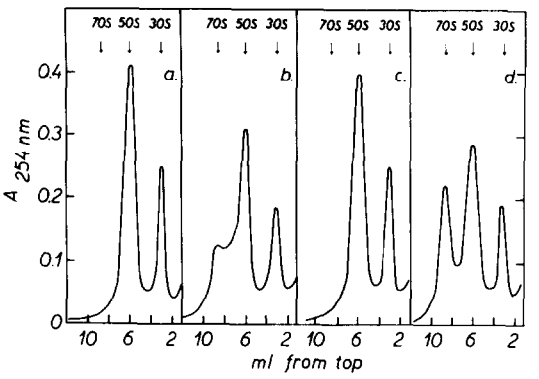

Fig. 4. Fractionation of AF. Sedimentation patterns of mixtures incubated at 55°. Standard assay conditions as described in Methods. Reactions were performed in the absence of AF (a) and with the addition of 10 μl of AF_{II} (b), 10 μl AF_I (c), or 10 μl AF_{II} + 10 μl AF_I (d).

incubated in the presence of the other subparticle and the association was measured afterwards by sucrose gradient centrifugation analysis (fig. 1). This experiment clearly showed that AF binds, at least functionally, to 30 S but not to 50 S subunits as association occurred only with the assumed (30 S–AF) complex (fig. 1c) suggesting either a low affinity of AF for the 50 S particle or a non-active (50 S–AF) complex. In our conditions the analysis by sucrose gradient centrifugation cannot distinguish between free 30 S subunit and the small subparticle with AF attached to it; both these species gave identical peaks in the ribosomal profiles.

After the resolution of the association process into partial reactions of binding and association we have been able to study some requirements of each separate step. It became clear that the partial reaction of association is not temperature dependent between 0° and 40° (fig. 2). However, it decreased at higher temperatures, possibly due to a relative instability or a partial inactivation of the isolated complex (30 S–AF) when heated above 40° without an excess of free AF.

Since the association step was maximal at low temperature, we have investigated the temperature dependency of the binding followed by association at 0° (fig. 3). Similar results, though with somewhat different yields, were obtained whether or not the (30 S–AF) complex had been isolated by gel filtration after the binding. In the first case the association amounted to 50% whereas in the second was 70–100% of the association values obtained by incubating all components together. These results could indicate that the presence of free AF in excess would stabilize the complex (30 S–AF) by shifting the equilibrium towards the binding and would also increase the saturation level of binding sites for AF on the 30 S subunit. On the other hand the temperature curve of the binding is very similar to that of the whole association process (fig. 3), although the particular absolute values are not identical probably due to the different preparations and experiments.

We have ruled out the possibility that the temperature dependent step was some conformational change of the free 30 S particles required to promote subsequent reactions, because the incubation of the small subunit at 55° followed by addition of AF and 50 S subparticles at 0° gave little or no association (M.G.P., unpublished results).

When AF was submitted to gel filtration through Sephadex G-25 it separated into two components: the excluded fraction AF_I and the included AF_{II} which act in different steps of the association process. Fig. 4 shows that fraction AF_{II} can induce the association of subunits to an "intermediate" or unstable form which appears as a shoulder in the sucrose gradient ribosomal profiles (fig. 4b). This peak could be the result of a partial dissociation during centrifugation [21] or a shift of the dynamic equilibrium between couples and

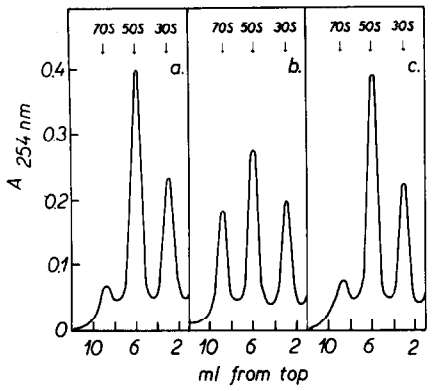

Fig. 5. Ribosomal binding of fraction AF_{II}. Sucrose gradient sedimentation profiles of subunits submitted to association in two steps (a) Control mixture without AF incubated at 55°. (b) Binding of 30 S particles and AF_{II} at 55°, and filtration through Bio-Gel were carried out as in fig. 1; after addition of 50 S subunits and AF_I (40 μg protein) the partial reaction of association was done at 0°. (c) Similar to (b), but the binding was performed with 50 S particles and AF_{II} followed by the second step after addition of 30 S subunits + AF_I.

free subunits [22]. On the other hand fraction AF_I, with no activity by itself*, is able to produce a relatively stable form of 30 S–50 S couple, when added after or together with fraction AF_{II} (fig. 4c and 4d).

Further experiments have indicated that AF_I is sensitive to trypsin digestion whereas AF_{II} is resistant to the same treatment (N.S.G., unpublished results).

Fraction AF_{II} binds readily to 30 S but not to 50 S particles (fig. 5b and 5c). It was found that this binding is temperature dependent in the same way as described above for the unfractionated AF.

Other preliminary studies have shown that AF_I is able to complete the association at 0° as well as at higher temperatures. AF_I apparently does not bind to any of the free subunits; however it might become attached to the (30 S–AF_{II}) complex.

From our results we conclude that the association of ribosomal subparticles is accomplished in two steps: a) binding of AF to 30 S subunit and b) formation of 30 S–50 S couples.

Fractionation of AF by gel filtration gave a relatively high molecular weight protein (AF_I), and a low molecular non-protein component (AF_{II}). The latter fraction has the binding properties of the complete AF and is able to induce the association to an "intermediate" or unstable form of 30 S–50 S complex, which can be then converted into the final product of the association process through the action of AF_I.

* Some preparations of AF_I contained a small amount of associating activity.

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